ADM

James R. Randall Research Center

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Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Room 1061
Rockville, MD 20852

RE: Interim Final Rule –"Health Claims: Plant Sterol/Stanol Esters and Coronary Heart Disease" (Docket Nos. 00P-1275 & 00P-1276); 65 Fed. Reg. 54686 (Sept. 8, 2000) and 66 Fed. Reg. 50824(Oct. 5, 2001)

Dear Sir or Madam:

The Archer Daniels Midland Company (ADM) submits the following comments in furtherance of ADM's comments to this Docket dated November 22, 2000, and in response to the Food and Drug Administration's (FDA) October 5, 2001 Federal Register notice reopening the comment period for the "Interim Final Rule for Plant Sterol/Stanol Esters and Coronary Heart Disease" (66 FR 50824).

Within the scope of the information requested by the FDA in its October 5, 2001 Notice, ADM provides the following comments and new information in support of the following actions:

1. The expansion of the Interim Final Rule to include unesterified (i.e., "free") sterol/stanols within the definition of the substance eligible for the coronary heart disease (CHD) health claim.

2. The expansion of the categories of the food listed in the Interim Final Rule as eligible to bear the plant sterol/stanols (free and esterified) CHD health claim, to include health bars, health drinks, and yogurt-type products.

Furthermore, in response to other specific issues raised by FDA, these comments will also address: (1) the daily intake levels of plant sterol/stanols (free or esterified) necessary to reduce the risk of CHD; (2) the effect of plant sterol/stanols on Vitamin A nutrition (impact on plasma carotenoids); and (3) and the safety of sterol/stanols during pregnancy.

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Finally, in support of the expansion of the food categories eligible for the CHD health claim and in furtherance of November 22, 2000 comments, we have attached, as an Appendix to these comments, the stage of development of an analytical method for the measurement of plant sterols (free and esterified) in different food matrices such as margarine spread, salad dressing, yogurt, orange drinks and health bars.

A. Eligibility of Unesterified Plant Sterols and Plant Stanols for the Health Claim

1. The Combined Weight of the Studies Previously Referenced by FDA and Data From Subsequent Studies Provide Ample Support for the Efficacy of Unesterified Plant Sterols for the CHD claim

The totality of the publicly available scientific evidence (i.e., studies on both esterified and unesterified sterols) unequivocally supports the ability of unesterified plant sterols to lower serum cholesterol and subsequently lower the risk of CHD. As noted in the preamble to the Interim Final Rule, plant sterol esters are hydrolyzed to free (unesterified) sterols and fatty acids in the gastrointestinal tract (65 FR 54690). The petitioner seeking the CHD health claim referred to the unesterified sterols as the "active moiety"[1] of the plant sterol esters, and sought to rely upon studies conducted on free sterols to support the efficacy of the sterol esters. *Id.* FDA agreed with the "active moiety" determination and "concluded that studies of the effectiveness of free plant sterols in blood cholesterol reduction are relevant to the evaluation of the evidence in the plant sterol esters petition." *Id.* Conversely, in light of the predictable conversion of sterol esters to free sterols in the gut, the studies using sterol esters that are referenced in the preamble to the Interim Final Rule are likewise "relevant" to the evaluation of the effectiveness of unesterified plant sterols in blood cholesterol reduction.

The weight of the evidence referenced in the preamble to the Interim Final Rule and summarized below has led to significant scientific consensus, as recognized by the National Cholesterol Education Program's ATP III report which makes no distinction between unesterified and esterified forms of plant sterols [1]. In fact, the preamble to the Interim Final Rule notes that the effects of various fat-based food matrices do not raise concerns regarding the efficacy of esterified <u>and unesterified</u> plant sterols. Specifically, the preamble states:

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[1] The term "active moiety" is more commonly used in the context of drug products where it is defined as "the molecule or ion, excluding those appended portions of the molecule that cause the drug to be an ester, salt (including a salt with hydrogen or coordination bonds), or other noncovalent derivative (such as a complex, chelate, or clathrate) of the molecule, <u>responsible for the physiological or pharmacological action of the drug substance</u>." 21 C.F.R. § 314.108(a) (emphasis added).

> Plant sterols (esterified or free) were tested in either a spread, margarine or butter carrier and produced fairly consistent results regardless of the food carrier and apparent differences in processing techniques. Given the variability of amounts and of food carriers in which plant sterols and plant sterol esters were provided in the diets studied, the response of blood cholesterol levels to plant sterols appears to be consistent and substantial, except for plant sterols from sheanut oil and rice bran oil. (65 FR 54701).

In addition to the publications cited in the Interim Final Rule supporting the efficacy of unesterified sterols in lowering serum cholesterol or inhibiting cholesterol absorption, subsequent publications have appeared in publicly available scientific literature further supporting this premise. The following descriptions of publications subsequent to the Interim Final Rule express cholesterol changes as relative reductions (in percentages) compared to the control groups unless otherwise indicated.

Vissers et al. [2] described the effect of unesterified plant sterols from rice bran oil on serum lipoprotein levels in humans. In this study, normocholesterolemic subjects consumed a portion of margarine (29 g of fat) containing 2.1 g of plant sterols/d for 3 weeks. Significant reductions in serum total cholesterol of 4.5% and LDL cholesterol of 8.5% were observed in subjects consuming the plant sterols. Plant sterols from rice bran oil used in this study were comprised of 50% 4-desmethylsterols (primarily β-sitosterol and campesterol) and 50% 4,4-dimethylsterols (primarily cycloartenols), therefore providing 1 g of 4-desmethylsterols/d. The authors concluded that the observed hypocholesterolemic effect of rice bran oil plant sterols was probably due to the 4-desmethylsterols content, a theory that the authors supported by numerous citations of publicly available scientific studies in humans.

Volpe et al. [3] demonstrated the ability of unesterified plant sterols from soybean to lower serum cholesterol in mildly hypercholesterolemic humans. Soybean plant sterols were administered as part of a low-fat yogurt-based drink (~2 g of fat) which delivered 1.1 g of sterols/d. Following 4 weeks of intervention, subjects consuming soybean plant sterols showed 4.4% and 6.2% reductions in serum total and LDL cholesterol levels, respectively. A subset of patients received 2 g sterols/d for an additional 4 weeks which showed a further decrease in serum total and LDL cholesterol levels; however, control values for these serum parameters were not provided so an accurate assessment of the cholesterol changes cannot be made.

In addition, ADM has attached, as Appendix A, the results of an unpublished clinical trial describing the effect of unesterified plant sterols in lecithin micellar form. The lecithin and plant sterol micelle was formulated in a diet beverage (30 ml) at a dose of 300 mg soybean plant sterols and 300 mg lecithin (0.2 g of fat). Over a 4-week intervention period, normocholesterolemic to mildly hypercholesterolemic subjects consumed the beverage 3 times/d for a total dose of 900 mg plant sterols/d. Inclusion of plant sterols resulted in lowering serum total and LDL cholesterol levels by 6% and 11%, respectively. The impact of plant sterols and stanols in micellar form on cholesterol absorption inhibition in humans has been substantiated by supporting information included in Appendix B and Ostlund et al. [4]. The relevancy of cholesterol absorption inhibition centers on this phenomenon being recognized as a primary mechanism of plant sterols in the small intestine.

As we noted in our November 22, 2000 comments to this docket, the scientific evidence reviewed and discussed in the preamble to the Interim Final Rule provides at least as much evidence of efficacy for the unesterified forms of sterols as it does for the esterified forms that were determined to be effective. That evidence, combined with the additional data summarized above and in the attached appendices, demonstrates significant scientific agreement that the CHD claims for unesterified plant sterols are supportable in accordance with requirements of Section 403(r) of the Federal Food, Drug, and Cosmetic Act (FDCA).

2. *The Scientific Evidence Justifies Expansion of the CHD Claim to Health Drinks, Health Bars, and Yogurt Products That Contain Plant Sterols*

The updated information regarding unesterified plant sterols provided in these comments, combined with the clinical data described in the preamble to the Interim Final Rule, support the efficacy of unesterified (and esterified) plant sterols in numerous low-fat food matrices. However, ADM is seeking expansion of the Interim Final Rule to include health drinks, health bars, and yogurt-type products containing the requisite amount of plant sterols (free or esterified) because these foods have currently met the agency's standards for inclusion in the CHD health claim regulation.

The current version of the Interim Final Rule authorizes the CHD health claim for spreads and dressings for salads that contain the requisite amount of plant sterol esters, while the plant stanol ester containing foods that may bear the health claim include spreads, dressings for salad, snack bars, and dietary supplements in soft gel form. See Interim Rule §101.83(c)(2)(iii)(A)(1). However, in the preamble to the Interim Final Rule, FDA stated that it will consider broadening the category of sterol containing foods eligible for the health claim provided that: (1) such foods are demonstrated to be "safe and lawful"; and (2) a validated analytical method is available that will accurately determine the amount of plant sterol esters within the food (65 FR 54707-08; see also 21 C.F.R. § 101.14(b)(3)(ii)). As described below, ADM has met both of these requirements with respect to health drinks, health bars, and yogurt-type products.

A conventional food is considered "safe and lawful" if it is generally recognized as safe (GRAS), listed as a "food additive," or authorized by a prior sanction issued by FDA. At the time that the Interim Final Rule was published, the petitioner seeking the health claim, Lipton, had provided the agency with information concerning the firm's GRAS determination for plant sterol esters in vegetable spreads and dressings for salads (See 65 FR 54688-89). Since that time, however, additional information on the GRAS status of other foods containing plant sterols has been submitted to the agency through the "GRAS Notification" process. On November 22, 2000, ADM submitted a "GRAS Notice" to FDA informing the agency of ADM's determination that plant sterols are GRAS, through scientific procedures, for use as an ingredient in vegetable oil spreads, dressings for salads, health drinks, health bars, and yogurt-type products at a level of 1 gram per serving (See GRAS Notice No. 000061). FDA responded to ADM's GRAS Notice on April 18, 2001, informing the company that "the agency has no questions at this time regarding ADM's conclusion that the ingredients plant sterols and plant sterol esters are GRAS under the intended conditions of use [i.e., spreads, dressings for salads, health drinks, health bars, and yogurt-type products]" (Letter dated April 18, 2001, from A. Rulis to M. Empie). Thus, health drinks, health bars, and yogurt-type products that contain up to 1 gram per serving of plant sterols (measured as free sterol) are GRAS and therefore "safe and lawful" under FDA's GRAS Notification policy (See 62 FR 18938 (Apr. 17, 1997)).[2]

With respect to the requirement for a validated analytical method, ADM has employed a general method in our laboratories to determine plant sterols in different food matrices (See Appendix C). Our preliminary results indicate that it is feasible to measure these compounds in different matrices. However, as it can be observed in the percent sterol recovery in the different food samples, each particular food matrix presents a different challenge, particularly regarding the sterol extraction. While we recognize that additional developmental work is needed to optimize this methodology, we expect that process to be completed within the next few months. Therefore, we believe that the Interim Final Rule can be expanded to include health drinks, health bars, and yogurt-type products with the full expectation that the remaining minor issues regarding the analytical methods for these products will be resolved in the near future.

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[2] We also note that FDA sent "no objection" letters to Cargill and Proctor & Gamble in response to GRAS Notices for the use of phytosterol esters in vegetable oil spreads, dressings for salads, bars, yogurt, and vegetable oil. See FDA Responses to GRAS Notices GRN 000048 and GRN 000053, dated November 27, 2000 and December 20, 2000, respectively.

B. Daily Intake Levels Necessary to Reduce the Risk of CHD

ADM supports the minimum daily intake level of 1.3 g plant sterol esters/d established in the Interim Final Rule. However, we believe that the regulation would benefit from the inclusion of a daily intake level expressed in terms of the equivalent dose of unesterified plant sterol (i.e., 0.8 g/d).

Because the unesterified plant sterol is the acknowledged "active moiety" that confers the health benefit, we also suggest that the level of sterol esters and unesterified sterol be expressed using a standardized "sterol equivalents" measure. Such a measure would reflect the amount of active material and provide a clear mechanism for monitoring and identifying sterol levels in food products, regardless of the form of the ingredient. Sterol equivalents would allow for standardized reporting and analytical methodology used for their determination. At the same time, clearly stating the corresponding amounts of the active moiety (free sterols) would provide a means for consumers to accurately select sterol-enriched food products.

The Agency has raised a question with regard to the equivalence of effective dose levels between plant sterols and plant stanols. ADM believes that plant sterols and plant stanols at high dosage levels, those above 2 g/d of ester forms, provide similar reductions in serum total and LDL cholesterol when directly compared. At this point in time, the literature is inconclusive with regard to discriminating between plant sterol esters and plant stanol esters consumed at dosage levels below 2 g/d. This notion is further supported by recently published data.

In Jones et al. [5], mildly hypercholesterolemic individuals consumed margarine (23 g of fat) with 1.84 g plant sterol esters/d (equivalent to 1.13 g unesterified plant sterols/d) or with 1.84 g plant stanol esters/d (equivalent to 1.13 g unesterified plant stanols/d) for 3 weeks. While significant reductions in serum total and LDL cholesterol levels were observed in both the plant sterol esters group and the plant stanol esters group (total: -9.1% sterol vs. -5.5% stanol; LDL: -12.9% sterol vs. -7.9% stanol), the effects in lowering cholesterol levels were significantly greater in the group that received the plant sterol esters. However, both groups lowered cholesterol with statistical significance.

Data on consuming higher levels of plant stanol esters indicate that the amount of 3.4 g/d of the current health claim amount is effective and that the value is likely excessive. However, the data for establishing a precise minimum effective amount of plant stanol esters has yet to be determined.

Additionally, ADM believes the total scientific evidence does not support any increase in the minimum daily intake level of plant sterol esters to establish harmonization with the minimum daily intake level of plant stanol esters. This scientific evidence establishes the consistency of the plant sterol esters in significantly lowering serum total and LDL cholesterol. With regard to sterols and their esters, recent data continues to support for the efficacy of plant sterol esters at a minimum sterol equivalent of about 0.8 g/d.

In Maki et al. [6], mild-to-moderate hypercholesterolemic subjects consumed 1.1 g plant sterol esters/d (equivalent to 0.7 g unesterified plant sterols/d) as part of a low-fat margarine for 5 weeks. Significant reductions in serum total and LDL cholesterol were observed (-5.2% and -7.6%, respectively). In a group of subjects consuming 2.2 g plant sterol esters/d (equivalent to 1.4 g unesterified plant sterols/d), no significant further lowering in serum cholesterol was observed due to the increased dose of plant sterol esters above 1.1 g/d.

C. Eligibility of Mixtures of Plant Sterols and Plant Stanols for the Health Claim

The sources of publicly available scientific literature show that ~60:20:20 ratio of β-sitosterol: campesterol: sitostanol and the corresponding mixture of esters have the ability to lower serum cholesterol [7, 8]. However, the latter reference used an intervention period of only 10d. The effective dose for the ~60:20:20 ratio of β-sitosterol:campesterol:sitostanol appears to be in the upper range of sterol dose compared to the unesterified and esterified forms of plant sterols and plant stanols. Precise data on the effectiveness of varying ranges of β-sitosterol: campesterol: sitostanol mixtures in humans is limited. The data on mixtures of plant sterols and plant stanols is insufficient to establish superiority of a particular mixture ratio compared to solely plant sterols or plant stanols.

D. Issues Regarding Safe of Use Plant Sterol/Stanol Esters in Foods and Advisory Label Statements

1. Effect of Phytosterols on Plasma Carotenoids and Impact on Vitamin A Nutrition

As noted in FDA's October 5, 2001 Federal Register notice, one of the concerns that has been raised, most notably by the European Commission (EC), concerning the consumption of sterols has been the impact on the plasma levels of carotenoids. While it is true that consumption of plant sterols tends to decrease the plasma levels of certain carotenoids, when taken in light of the factors discussed below, this decrease does not present any safety or public health concerns.

Several human studies have shown either an insignificant decrease in the plasma carotenoid levels [9-11] or a significant decrease that becomes insignificant when adjusted to changes in the plasma lipid profile [9, 12]. However, when looking at particular carotenoids, there is a consistent and clear tendency for a decrease, even after correcting for lipid changes, in the plasma levels of α and β-carotenes and lycopene [13, 14]. Changes in the plasma levels of other carotenoids or other fat soluble vitamins have been inconsistent [9]. It appears, then, that only the more hydrophilic carotenoids, such as α- and β-carotene and lycopene are affected by the ingestion of sterols. Various carotenoids need to be solubilized in the lipid core of the micelles in the gut lumen for proper intestinal absorption [15]. This is a somewhat similar mechanism for the effect of sterols on intestinal cholesterol absorption; therefore, it is likely that carotenoids are also affected and thus their plasma levels decrease. However, as described below, when one considers the factors relevant to plant sterol consumption, it becomes evident that this phenomenon has no nutritional or public health significance, particularly in the long term:

1 - Despite the decrease in carotenoid levels, such values are still within the normal range and even above what is considered the average normal values [15]. This can be illustrated by using the data from a Unilever study submitted to the Scientific Committee on Foods (SCF) of the European Union regarding the changes observed in β-carotene after 26 and 52 weeks of consumption by adults of a fat spread containing 8% (w/w) phytosterol esters [16]. For comparison, average and reference ranges β-carotene plasma values have been included as part of the following table:

CHANGES ON PLASMA β-CAROTENE AFTER ONE YEAR
CONSUMPTION OF STEROLS COMPARED TO NORMAL REFERENCE VALUES

Time	β-Carotene[a] (μmol/L)	Average Reference Value (M/F)[b] (μmol/L)	Reference Range[b] (5-95th percentile) (μmol/L)
Week 0	0.41 ± 0.22		
Week 26	0.32 ± 0.18		
Week 52	0.31 ± 0.19	0.22/0.28	0.09-0.91

a - Adapted from European Commission Report (Ref. [16]).
b - From Olson, J., 1999. (Ref. [15]); M: Adult males, F: Adult females

It can be observed in this investigation that after one year of phytosterol consumption by adult subjects, there is a 24.4% drop in plasma β-carotene from baseline. The β-carotene value, however, even at 52 weeks is above the plasma average reference value for both men and women in a normal population and well within what is considered a normal plasma β-carotene range (5- 95th percentile). No other levels of vitamins or other carotenoids were affected in this study. The same phenomenon has been observed in a more recent study by Maki et al. [6] in the United States. In this investigation, blood carotenoid levels remained within normal limits after the consumption by adult subjects of 1.1 g/day or 2.2 g/day of plant sterol esters for a period of five weeks.

2 - When consuming phytostanol esters, the actual plasma levels of Vitamin A (retinol), as well as of other fat soluble vitamins, are not altered [14]. In fact, the intestinal absorption of Vitamin A (as retinyl palmitate), and also the absorption of other fat soluble vitamins, is not affected when given to humans as part of a fat load test in the presence of stanol esters [17].

3 - The contribution of carotenoid to Vitamin A nutrition is very low. Thus, within the context of a diet containing adequate preformed Vitamin A, carotenoids are not needed by mammals and, therefore, cannot be considered essential [15]. In fact, the intestinal dietary absorption of carotenoids is only 10-50%. Furthermore, only a small portion of those absorbed are converted to retinol. Different carotenoids, depending on their molecular structure, have different biological activities. For practical purposes, the conversion of mixed carotenoids as they occur in the diet is considered to be only 1/12 of the preformed Vitamin A activity [15]. Thus, it can be assumed that a ~25% carotenoid loss (as in the previous Unilever study, Ref. [16]) really represents only a potential 2% contribution to Vitamin A nutrition (25 x 1/12) due to the intestinal absorption rate. In theory, this would be the portion that is being lost by the body due to the sterol consumption. Considering a RDI for Vitamin A of 800-1000 μg RE/ day for the American adult [18], this is only about 20 μg of dietary Vitamin A – an amount that can be easily compensated from dictary sources (e.g. one cup of unfortified whole milk has 101 μg of Vitamin A). Thus, when there is enough dietary preformed Vitamin A, this 2% is not critical.

4 - Vitamin A is stored in the liver and it is not easily depleted. Normal liver stores range from 20-300 μg/g liver [19]. Thus, an adult liver weighing 1.5 kg will contain about 240,000 μg (0.24 g) of stored Vitamin A (considering an average liver Vitamin A concentration of 160 μg/g). If the half life (t½) of stored Vitamin A is 128-156 days (average 142 days) it would take 14.2 months (more than 1 year) for liver stores to reach deficient levels (three half life equivalents before reaching <20 μg retinol/g liver) [19]. In fact, actual Vitamin A deficiency studies in adult humans have shown that it takes up to 2 years to reach a deficiency state when consuming a Vitamin A depleted diet [20]. It would be highly unlikely to find these severe conditions in a normal setting. Thus, a potential 2% loss on Vitamin A nutrition, due to the

observed decrease in carotenoids, becomes insignificant when considering the depletion rate of Vitamin A.

5 - The observed changes in plasma levels of carotenoids (up to 24.4% drop in β-carotene in adults after one year of sterol consumption) or fat soluble vitamins have never been associated with any functional alterations or pathological condition. Thus, these changes in adult individuals can be considered clinically unimportant and therefore of no significant health concern.

6 - Vitamin A deficiency is not a public health concern in the United States [21]. On the contrary, excessive carotenoids intake has recently been a target for reduction in developed areas such as Europe. Therefore, in general, there is no "at risk" population group in the developed world that could easily become deficient in this vitamin. From the dietary perspective, in the United States, Vitamin A sources are primarily preformed Vitamin A from fortified foods such as fortified milk, yellow margarines, breakfast cereals and from other dietary sources like liver, green/yellow vegetables and eggs.

Given the above scientific analysis, it can be concluded that the observed decrease in carotenoids would not represent a public health risk for the U.S. population.

2. *Consideration of Serum Cholesterol Modulation during Pregnancy*

Another potential issue that has been raised by the EC and noted by FDA is the consumption of sterols during pregnancy. Specifically, there could be a possibility of lowering serum cholesterol in the mother and the developing fetus to levels which might be of potential concern. Based on currently available scientific literature, there appears to be little risk to either the mother or the developing fetus if plant sterols are consumed during pregnancy. However, we would agree that it is prudent for a woman to consult her doctor concerning plant sterol consumption during pregnancy.

The literature to address this question is somewhat limited. Data provided on the Centers for Disease Control (CDC) website indicate that mean serum cholesterol levels in women rise from 184 mg/dl for the age group 20-34 years to 217 mg/dl for women aged 45-54 years. However, during pregnancy, it is generally observed that maternal cholesterol levels rise 25 to 40%, with the rise increasing steadily through the third trimester [22]. Following parturition, cholesterol levels fall rapidly [23]. With regard to the fetus, only a few measurements of fetal serum cholesterol have been made and these values increased by trimester. Furthermore, Kesteloot et al. [24] have shown that a significant, independent correlation exists between fetal cord blood cholesterol and the cholesterol level of the mother.

CONCLUSIONS:

For the reasons set forth above, ADM urges FDA to amend the Interim Final Rule for plant sterol/stanol esters and CHD health claim to include unesterified (free) sterol/stanol compounds and to expand the food categories covered by the claim by adding yogurt type products, health drinks and health bars. Furthermore, the data analysis provided herein on the impact of sterol/stanols (free or esterified) on Vitamin A nutrition shows that the observed decrease in plasma carotenoid levels has no public health significance. In regard to pregnancy and the developing fetus, it appears that there is little risk if sterols are consumed during that period.

Finally, our preliminary laboratory results have confirmed the feasibility of developing a plant sterol analytical method to measure such compounds from different food matrices. Although, work is still needed to adapt and refine the analytical techniques to each particular food, we expect those refinements to be completed in the near future.

Sincerely,

Mark W Empie

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Vice President,
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MWE/jm

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APPENDICES

A - Clinical Study; Impact of Soy Sterols:Lecithin Micelle on Serum Cholesterol Levels (unpublished).

B - Clinical Study; Acute Cholesterol Absorption in Humans (sterols data unpublished).

C - Analytical Method for the Determination of Free or Esterified Plant Sterol in Different Food Matrices

Appendix A. (with permission)

Clinical study measuring the impact of soy sterols:lecithin micelle on serum cholesterol levels

Investigator: Curtis Spilburg (supported by NIH grant 1R43-HL62780)
Site: Lifeline Technologies, Chesterfield, MO

Serum cholesterol was measured in human subjects to examine the effects of soy sterols in lecithin micelle. The study followed a randomized, parallel, double-blind, placebo design. Subjects (n=26) adhered to the AHA Step I diet for 4 weeks prior to randomization then received either placebo or control for another 4 weeks while continuing on the AHA Step I diet. Subjects received 30 mL of a flavored diet beverage containing 300 mg sterols in micelle form (active) or lecithin micelle (placebo) three times per day for a total dose of 900 mg sterol. Subjects agreed to participate by signing an IRB-approved consent form. Subjects had an average serum total cholesterol of 188 mg/dl and LDL-cholesterol of 119 mg/dl.

Results:

The soy sterols group had significantly lower total (-6.3%; $p<0.05$) and LDL (-11.4%; $p<0.04$) cholesterol beyond the cholesterol levels of the placebo group. HDL cholesterol was lower in the soy sterols group but not significantly (-5.1%; $p<0.2$).

Appendix B. (with permission)

Acute Cholesterol Absorption in Human Subjects

Investigator: Richard Ostlund, Jr. (supported by NIH grant HLR01-50420)
Site: Washington University, St. Louis, MO

The absorption of deuterated cholesterol was measured in human subjects to examine the inhibition of uptake effects by soy sterols:lecithin micelle. Each subject served as their own control and received the test substances 30 minutes prior to a controlled test meal containing a specific amount of total cholesterol containing deuterated cholesterol tracer.

Design:

Baseline -- 2 week washout -- **Placebo lecithin micelle** -- 2 week washout -- **Sterol:lecithin micelle**
OR Sterol:lecithin micelle **OR Placebo lecithin micelle**

Results:

Sterol	Sterol Dose	Cholesterol absorption
Sitostanol	1000 mg	-10%
Sitostanol:lecithin	100 mg	- 5%
"	300 mg	-33%
"	700 mg	-35%
Soy Sterols:lecithin	420 mg	-21%

Appendix C.

Determination of Sterols in Foods Enriched with Sterols/Sterol Esters by Gas Chromatography

1.0 Overview.

This method is applicable to a variety of foods containing sterols and sterol esters. Samples are refluxed using 20% potassium hydroxide in methanol to convert any sterol esters to free sterols. Sample extracts are acidified to pH <3 using concentrated hydrochloric acid, partitioned into methylene chloride, and analyzed for the free sterols without further derivatization using a gas chromatograph equipped with a flame ionization detector. Quantitation may be performed using either stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene) as an external standard or epicoprostanol (5β-cholestan-3α-ol) as an internal standard with its response correlated to stigmasterol.

2.0 Equipment.

Gas chromatography system equipped with a flame ionization detector, Agilent Model 6890 or equivalent
J&W Scientific DB-5HT GC column, 30 m length, 0.32 mm id, 0.1 μm film thickness
Thermolyne Model Cimarec 3 block heater
ESGE Model 133/1281-0 bio homogenizer

3.0 Reagents.

Methanol, HPLC grade
Hydrochloric acid, trace metal grade, 35-38% w/w
Potassium hydroxide, Certified A.C.S.
Sodium chloride, Certified A.C.S.
Methylene chloride, A.C.S. spectrophotometric grade
Sodium sulfate, Certified A.C.S.
Magnesium sulfate, Certified A.C.S.

4.0 Standards.

Epicoprostanol (5β-cholestan-3α-ol), CAS No. 516-92-7, Sigma Lot No. 109H4081, purity = 95%, FW 388.7
Stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene), CAS No. 83-48-7, Sigma Lot No. 59F0638, purity = 96%, FW 412.7

5.0 Safety.

Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheets for all chemicals used in this procedure. Methylene chloride and hydrochloric acid should be handled under a fume hood using solvent-resistant gloves. The 20% solution of potassium hydroxide in methanol may react violently in the presence of excess water. During addition of strong bases or acids, glassware openings should be directed toward the back of the fume hood to avoid potential exposure.

6.0 Standards/Solution Preparation.

Stigmasterol calibration solutions are prepared in methylene chloride at concentrations ranging from 10 to 0.1 mg/mL.

Epicoprostanol fortification solutions are prepared in hexane at a target concentration of 10 mg/mL.

Epicoprostanol and stigmasterol mixed calibration solutions are prepared in methylene chloride.

Potassium hydroxide in methanol (~20%) is prepared by adding 400 g of potassium hydroxide to a 2 L bottle containing 1800 mL of methanol.

Saturated sodium chloride is prepared by adding excess sodium chloride to water.

7.0 Procedure.

1. This method is applicable to several matrices. Individual serving sizes, levels of added sterols, and sample sizes that were used to develop the method are provided:

Matrix	Serving Size	Sterol Esters (mg/serving)	Free Sterols (mg/serving)	Sample Size
Salad Dressing	33 g	400	400	8.25 g
Margarine Spread	15 g	1031	429	3.75 g
Orange Drink	250 mL	422	450	25 mL
Yogurt	240 g	434	456	24 g
Health Bar	8 g	445	463	2 g

2. Excess water will interfere with the procedure so samples high in moisture (orange drink, yogurt) are freeze-dried prior to extraction.

3. Transfer the appropriate mass of sample to a 250 mL flat-bottom flask. Add 1.0 mL of epicoprostanol standard solution (10 mg/mL hexane) to each sample.

4. Add 50 mL of 20% potassium hydroxide in methanol plus several boiling chips to each flask. Attach a condenser and reflux for 1.5 hours. If a gelatinous film develops in the sample due to the presence of gum additives, homogenize with an electric hand-held unit (ESGE Model 133/1281-0 bio homogenizer).

5. Allow to cool, and neutralize to pH <3 using concentrated hydrochloric acid (approx. 16 mL).

6. Quantitatively transfer the extracts to 250-mL separatory funnels and add 50 mL of saturated sodium chloride. Partition each sample into 3 x 25 mL of methylene chloride.

7. Combine the methylene chloride fractions, dry with magnesium sulfate or sodium sulfate, and bring to volume with methylene chloride in a 100-mL volumetric flask.

8. Analyze the sample extracts using gas chromatography.

Injection volume	1 µL		
Injector mode	split mode 70:1		
Inlet temp.	375 C		
Column	J&W Scientific DB-5HT, 30 m length, 0.32 mm id, 0.1 µm film thickness		
Column temp.	Rate	Next Temp	Hold Time
	Initial	170 C	5 min
	3 C/min	300 C	0 min

	80 C/min	375 C	10.73 min
Carrier flow	Helium at 3 mL/min constant flow		
Detector temp.	375 C		
H_2 flow	40 mL/min		
Air flow	450 mL/min		
Makeup flow (N_2)	50 mL/min		
Run Time	60 min		

8.0 Calculations

Quantitation of brassicasterol, campesterol, campestanol, sitosterol and sitostanol may be performed using either external or internal standard calibration.

Calibration based on External Standards: (optional)

The external calibration curve is calculated as:

$$Amt_{STIG} = f\ (Area_{STIG})$$

Where:
Amt_{STIG} = the amount of stigmasterol calibration standard
f = the linear equation of calibration
$Area_{STIG}$ = the area of stigmasterol calibration standard

The concentration of each sterol in the sample is determined by:

$$Amt_{STEROL} = f\ (Area_{STEROL})$$

Where:
Amt_{STEROL} = the amount of sample sterol
f = the linear equation of calibration (stigmasterol)
$Area_{STEROL}$ = the area of the sample sterol

Calibration based on Internal Standards:

The ratio of stigmasterol amount to epicoprostanol (ISTD) amount is plotted versus the ratio of stigmasterol area to epicoprostanol (ISTD) area. Sample area ratios of sterol/epicoprostanol are then substituted for the stigmasterol/epicoprostanol area ratio.

The calibration curve is calculated as:

$$\text{Amt Ratio}_{STIG/EPI} = f\ (\text{Area Ratio}_{STIG/EPI})$$

Where:
Amt $\text{Ratio}_{STIG/EPI}$ = the amount ratio of stigmasterol/epicoprostanol calibration std
f = the linear equation of calibration
Area $\text{Ratio}_{STIG/EPI}$ = the area ratio of stigmasterol/epicoprostanol calibration std

The concentration of each sterol in the sample is determined by:

$$\text{Amt Ratio}_{STEROL/EPI} = f\ (\text{Area Ratio}_{STEROL/EPI})$$

Where:
Amt_{SAM} = the amount of sample sterol
f = the linear equation of calibration (stigmasterol)
Area_{SAM} = the area of the sample sterol

10.0 Method Data

Stigmasterol yielded a linear calibration curve from 10 to 0.1 mg/mL. The calibration curve was forced through zero and had a correlation coefficient of >0.99.

The Limit of Detection for stigmasterol was estimated to be 0.05 mg/mL.

Determination of Sterols in Foods Enriched with Sterols/Sterol Esters by Gas Chromatography

Calibration Method: Internal Standard

	Serving Size	Free Sterol or Equivalent Added Amount	Free Sterol Detected Amount Mean	Std. Dev.	Recovery[a]
		(mg/serving)	(mg/serving)	(mg/serving)	
Salad Dressing	33 g				
Control		NA	41		
Sterol Ester		392	513	45	120.3%
Free Sterol		399	534	19	123.5%
Orange Drink	250 mL				
Control		NA	28		
Sterol Ester		422	590	79	133.3%
Free Sterol		450	531	39	111.8%
Yogurt	240 g				
Control		NA	1		
Sterol Ester		434	281	45	64.4%
Free Sterol		456	319	10	69.8%
Margarine spread	15 g				
Control		NA	48		
Sterol Ester		1031	1138	85	105.7%
Free Sterol		429	477	55	99.8%
Health Bar	8 g				
Control		NA	4		
Sterol Ester		445	457	7	101.8%
Free Sterol		463	512	23	109.8%

[a] Recovery (%) = [(Free Sterols in sample - Free Sterols in control) / Free Sterols Added] * 100